

EXHIBIT F

Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens

(human papillomavirus/LAMP-1/recombinant vaccinia)

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Communicated by John W. Littlefield, Johns Hopkins University, Baltimore, MD, August 3, 1995

ABSTRACT The presentation of antigenic peptides by major histocompatibility complex (MHC) class II molecules to CD4⁺ T cells is critical to the function of the immune system. In this study, we have utilized the sorting signal of the lysosomal-associated membrane protein LAMP-1 to target a model antigen, human papillomavirus 16 E7 (HPV-16 E7), into the endosomal and lysosomal compartments. The LAMP-1 sorting signal reroutes the antigen into the MHC class II processing pathway, resulting in enhanced presentation to CD4⁺ cells *in vitro*. *In vivo* immunization experiments in mice demonstrated that vaccinia containing the chimeric E7/LAMP-1 gene generated greater E7-specific lymphoproliferative activity, antibody titers, and cytotoxic T-lymphocyte activities than vaccinia containing the wild-type HPV-16 E7 gene. These results suggest that specific targeting of an antigen to the endosomal and lysosomal compartments enhances MHC class II presentation and vaccine potency.

The presentation of antigenic peptides by major histocompatibility complex (MHC) class II molecules to CD4⁺ T cells is critical to the function of the immune system. CD4⁺ T cells are the major helper T-cell phenotype whose predominant function is to generate cytokines that regulate essentially all other functions of the immune response. CD4⁺ MHC class II restricted cells have also been shown to have cytotoxic capacity in a number of systems, including a response to fragments of the human immunodeficiency virus gp120 protein (1). CD4⁺ cells have also been shown to be of great importance in immune responses against several different murine (2, 3) and human (4) solid malignancies. Several mouse tumors that were transfected with syngeneic MHC class II genes have become very effective vaccines against subsequent challenge with wild-type (wt) class II negative tumors (5). For these reasons, there has been increased interest in developing strategies that will most effectively activate CD4⁺ MHC class II restricted cells against a given specific antigen (6).

Two major pathways by which antigens enter endosomal and lysosomal compartments for MHC class II presentation to CD4⁺ T cells have been described. The traditional pathway involves the phagocytosis or endocytosis of exogenous proteins into antigen-presenting cells (APCs), followed by degradation by acid proteases in the endosomal or lysosome-like compartments (7–9). A second pathway involves the processing of membrane proteins endogenously synthesized by APCs (1, 10). These membrane proteins are believed to enter endosomal and lysosomal compartments by internalization from the cell surface. In certain experimental systems, cytoplasmic proteins may also enter this endogenous MHC class II pathway (11, 12), but normally these antigens are preferentially routed for MHC class I presentation. In general, cytoplasmic or nuclear pro-

teins are degraded into peptides in the cytoplasm, which are then transported into the endoplasmic reticulum where they complex with newly assembled class I molecules on their way to the cell surface for presentation to CD8⁺ T cells (for review, see ref. 13).

We reasoned that a molecular approach that directly routes an antigen into the MHC class II processing and presentation pathway, such as endosomal and lysosomal compartments, might enhance its presentation to MHC class II restricted CD4⁺ T cells. The endosomal and lysosomal compartments are characterized by the presence of several compartment-specific membrane proteins. The lysosomal-associated membrane protein LAMP-1 is a type 1 transmembrane protein localized predominantly to lysosomes and late endosomes (14, 15). The cytoplasmic domain of LAMP-1 contains the amino acid sequence Tyr-Gln-Thr-Ile, whose structure conforms to Tyr-Xaa-Xaa-hydrophobic amino acid motif that mediates cell membrane internalization and possibly lysosomal targeting of several cell surface receptors (16–20). The intracellular targeting of LAMP-1 has been shown to be controlled by the amino acid sequence Tyr-Gln-Thr-Ile at the C terminus of its cytoplasmic tail (21, 22). We therefore engineered a chimeric gene encoding a model antigen linked to the transmembrane and cytoplasmic region of LAMP-1, which we hypothesized would target the antigen to the endosomal and lysosomal compartments.

We chose the human papillomavirus 16 E7 (HPV-16 E7) as a model antigen for two reasons: (i) HPV-16 E7 is a characterized cytoplasmic/nuclear protein (23–26) and (ii) immunological studies targeting HPV-16 E7 can potentially lead to development of therapeutic vaccines against HPV-associated malignancies (for reviews, see ref. 27). In this study, our data suggest that specific targeting of HPV-16 E7 to the endosomal and lysosomal compartments enhances MHC class II presentation and results in increased vaccine potency *in vivo*.

MATERIALS AND METHODS

DNA Constructs. For generation of the Sig/E7/LAMP-1 chimeric gene, DNA fragments encoding the signal peptide (N-terminal) of LAMP-1, the open reading frame of HPV-16 E7, and the transmembrane domain and cytoplasmic tail of LAMP-1 were amplified by PCR using high-fidelity *Pfu* polymerase (Stratagene). The primer sets were designed so that *Bgl* II and *Bam*HI restriction sites were generated at the 5' and 3' ends of the amplified fragments, respectively. The primer set for signal peptide of LAMP-1 was 5'-TTGAGATCTTATG-

Abbreviations: MHC, major histocompatibility complex; wt, wild-type; APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; LAMP-1, lysosomal-associated membrane protein; mAb, monoclonal antibody; HPV, human papillomavirus; pfu, plaque-forming units.

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GCGGCCCCC-3' and 5'-TTGGGATCCTCAAAGAGTGCTGA-3'. The primer set for HPV-16 E7 open reading frame was 5'-CCCAGATCTAATCATGCATG-3' and 5'-TATGGATCC-TGAGAACAGAT-3'. The primer set for the transmembrane and cytoplasmic domain of LAMP-1 was 5'-TCAAGATCT-TAACAACATGTTG-3' and 5'-TGTGGATCCCTTCCACA-CC-3'. The amplified DNA fragments were cloned sequentially into the unique *Bam*HI cloning site of the pCMVneo expression vector (28) downstream of the cytomegalovirus promoter.

Transfections and Immunofluorescence Stainings. Transfections and immunofluorescent stainings were performed as described (22). Mouse anti-HPV-16 E7 monoclonal antibody (mAb) (Triton, San Diego) was used to detect HPV-16 E7. Rat anti-mouse LAMP-1 mAb (1D4B) (14) was used to detect LAMP-1.

Proliferation Assay. Proliferation assays were performed following the protocol of Kruisbeek and Shevach (29). For *in vitro* assays, CBF1 mice were injected in the foot pad with 20 μ g of HPV-16 E7 peptide (aa 30–67) mixed with complete Freund's adjuvant. Lymphocytes were prepared by crushing peripheral lymph nodes from CBF1 mice 2 wk after *in vivo* priming. An aliquot of 3×10^5 cells was plated in triplicate in 96-well plates with decreasing dilutions of irradiated (5000 rad; 1 rad = 0.01 Gy) LB27 APCs stably transfected with E7, Sig/E7, or Sig/E7/LAMP-1. For *in vivo* assays, lymphocytes from peripheral lymph nodes of vaccinia-infected C57BL/6 mice or CD4-knockout C57BL/6 mice were mixed with decreasing dilutions of E7 30–67 peptides.

Recombinant Vaccinia Viruses. Recombinant vaccinia viruses were generated following the protocol of Earl and Moss (30–32). Plaque-purified recombinant vaccinia viruses were tested for the presence of HPV-16 E7 genome by PCR and for the expression of HPV 16 E7 protein by immunofluorescent stainings.

Cytotoxic T-Lymphocyte (CTL) Assays. Induction and measurement of CTL activity were performed using standard protocols (33). Splenocytes from BALB/c, C57BL/6, or CD4-knockout C57BL/6 mice were used for CTL assays. Splenocytes were harvested 2 wk after mice were infected with 10^7 plaque-forming units (pfu) of recombinant vaccinia by i.p. injection. Splenocytes were cocultured with mitomycin C-treated HPV-16 E7 containing syngeneic tumor cells (stimulators) for 6 days. CTL assays were performed in a standard 4-hr chromium release assay. Target cell-specific lysis was determined by subtracting each sample's % of wt target cell CD8-blockable lysis from its corresponding % of E7 target cell CD8-blockable lysis.

ELISA. The anti-HPV-16 E7 antibodies in the sera from recombinant vaccinia-infected C57BL/6 mice or CD4-knockout C57BL/6 mice (34) were detected by ELISA using microwell plates coated with synthetic E7 peptides (aa 30–67) or yeast-derived HPV-16 E7 protein prepared as described (35).

RESULTS

Generation of Chimeric Sig/E7/LAMP-1 Molecule. To target HPV-16 E7, a cytoplasmic and nuclear protein, to the endosomal and lysosomal compartments, it was first necessary to place a signal peptide at the N terminus of the protein to mediate translocation into the lumen of the endoplasmic reticulum. The transmembrane domain and cytoplasmic tail of LAMP-1 were placed at the C terminus of the E7 protein because these components are known to confer endosomal/lysosomal targeting (22). The DNA fragments that encode the N-terminal signal peptide sequence of LAMP-1, HPV-16 E7, and the transmembrane and cytoplasmic domains of LAMP-1 were amplified by PCR and sequentially cloned into a mammalian expression vector. Fig. 1 shows a diagram of the final construct, which was confirmed by DNA sequencing. The chimeric Sig/E7 is the intermediate product of Sig/E7/

LAMP-1. Sig/E7 was generated by placing the signal peptide of LAMP-1 at the N terminus of the HPV-16 E7.

Addition of LAMP-1 Signal Peptide, Transmembrane, and Cytoplasmic Portions onto HPV-16 E7 Protein Efficiently Reroutes E7 into the Endosomal/Lysosomal Compartment. Transfections and subsequent immunofluorescent stainings were used to determine expression and localization of wt and modified HPV-16 E7 protein. As expected, cells transfected with wt E7 showed homogeneous cytoplasmic/nuclear staining (Fig. 2B). In comparison, cells transfected with the chimeric Sig/E7/LAMP-1 construct displayed a vesicular pattern consistent with endosomal and lysosomal localization (Fig. 2A). To further confirm localization of the Sig/E7/LAMP-1 chimera to the endosomal and lysosomal compartments, we performed double labeling experiments of cells stably transfected with Sig/E7/LAMP-1 using antibodies against HPV-16 E7 and the N-terminal (or luminal) portion of endogenous LAMP-1, a well-characterized marker for the endosomal and lysosomal compartments. Colocalization of E7 and endogenous LAMP-1 was clearly visible (Fig. 3), indicating that the Sig/E7/LAMP-1 chimera was indeed targeted to the endosomal and lysosomal compartments (see Fig. 3 Insets). Controls omitting primary antibodies did not show specific staining (data not shown).

Modification of Endogenously Synthesized E7 with the LAMP-1 Sorting Signal Enhances Presentation to MHC II Restricted T Cells. To demonstrate that the Sig/E7/LAMP-1 chimeric protein efficiently enters the MHC class II processing

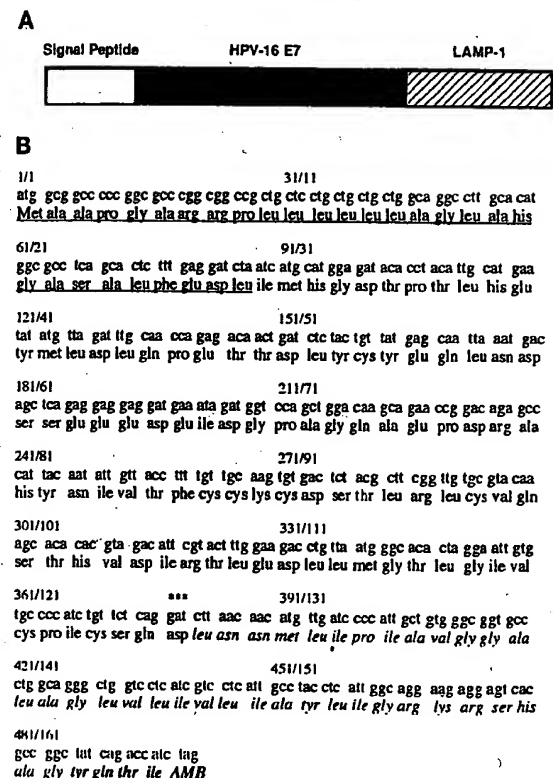


Fig. 1. Diagram and nucleotide sequence of the chimeric Sig/E7/LAMP-1 protein. (A) Diagram of chimeric Sig/E7/LAMP-1. The signal peptide is white, HPV-16 E7 is black, and the LAMP-1 transmembrane and cytoplasmic tail is hatched. (B) Nucleotide sequence of chimeric Sig/E7/LAMP-1. The signal peptide is underlined. The LAMP-1 transmembrane and cytoplasmic tail is in italics. The lysosomal targeting signal is in boldface. Asterisks (***) indicate the last amino acid of chimeric Sig/E7 protein (127 aa).

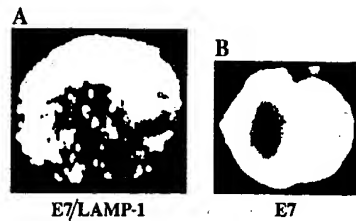


FIG. 2. Immunofluorescent staining of HPV-16 E7 and E7/LAMP-1 chimeric proteins in the transfected cells. Human embryonic kidney cells, 293S, were transiently transfected with pCMVneo/Sig/E7/LAMP-1 (A) or pCMVneo-16E7 (B) by calcium phosphate-DNA coprecipitation. 293S cells were fixed, permeabilized, and stained with mouse anti-HPV-16 E7 mAb followed by Texas red-conjugated goat anti-mouse secondary antibody (A and B). Note the positive vesicular patterns that were appreciated in cells transfected with Sig/E7/LAMP-1 recombinant plasmid (A). In contrast, diffuse cytoplasmic as well as nuclear stainings were noted in cells transfected with wt HPV-16 E7 plasmid (B). ($\times 500$.)

and presentation pathway, we assayed for stimulation of E7-specific MHC class II restricted proliferative responses. LB27, a B-cell hybridoma expressing I-A^{b,d} and I-E^{b,d} MHC

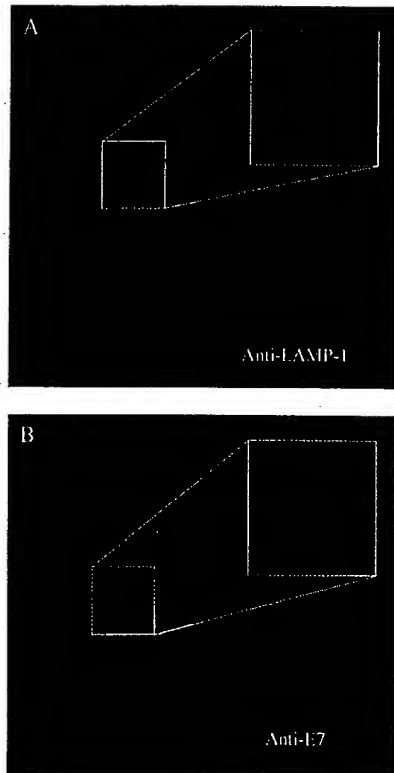


FIG. 3. Colocalization of E7 and LAMP-1 proteins in cell transfected with chimeric Sig/E7/LAMP-1 by double labeling immunofluorescent stainings. LB27 cells were stably transfected with pCMVneo/Sig/E7/LAMP-1. Double labeling immunofluorescent stainings were performed by first incubating transfected LB27 cells with the mouse anti-HPV-16 E7 mAb and the rat anti-mouse LAMP-1 mAb (1D4B) followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and Texas red-conjugated goat anti-rat IgG secondary antibodies. For detection of endogenous LAMP-1, Texas red fluorescent staining was noted (A). For detection of HPV-16 E7 protein, FITC fluorescent staining was noted (B). (Insets) Enlargements of the same compartments that were positive for E7 protein and endogenous LAMP-1. Controls that omitted primary antibodies did not show specific staining (data not shown). ($\times 125$.)

class II molecules as APCs, was transfected with either wt HPV-16 E7, Sig/E7, or Sig/E7/LAMP-1 genes in plasmids encoding neomycin resistance. G418-resistant clones were isolated by limiting dilution and tested for the presence of HPV-16 E7 or Sig/E7/LAMP-1 by PCR and immunofluorescent stainings (data not shown). HPV-16 E7-specific lymphocytes were generated by injecting the foot pad of CBF1 mice with HPV-16 E7 peptide (aa 30–67) emulsified in complete Freund's adjuvant. This HPV-16 E7 peptide has been reported to contain a T-helper cell epitope (aa 44–60) capable of being presented by multiple MHC class II alleles (36). The HPV-16 E7 peptide-primed lymphocytes showed specific proliferative responses to E7 44–60 and E7 30–67 (data not shown). Fig. 4 compares the ability of E7, Sig/E7, and Sig/E7/LAMP-1 transfected LB27 cells to stimulate E7 30–67-specific T cells. E7 and Sig/E7 transfectants fail to stimulate E7 30–67-specific T cells, consistent with the inefficient entry of intracellular proteins into the MHC class II processing pathway. In contrast, Sig/E7/LAMP-1 expressing LB27 cells efficiently stimulated HPV-16 E7 30–67-specific T cells. These results demonstrate that the Sig/E7/LAMP-1 chimeric protein is efficiently processed and presented in the MHC class II pathway. Virtually identical results were obtained when responder T cells were depleted of CD8⁺ cells, demonstrating that the enhanced proliferative response to Sig/E7/LAMP-1 transfectants is predominantly due to CD4⁺ activation (data not shown).

Modification of E7 with the LAMP-1 Sorting Signal Enhances *In Vivo* Immune Responses Induced by Recombinant Viral Vaccines. To measure the effect of LAMP-1 targeting of antigen *in vivo*, we selected the vaccinia virus system. Vaccinia virus has a large capacity for genetic insertion: (37). Since vaccinia immunization has been postulated to involve direct infection of APCs, it represented a good vaccine system to analyze the consequences of LAMP-1-mediated targeting of antigen *in vivo*. Plaque-purified recombinant vaccinia viruses were tested for the presence of the HPV-16 E7 and Sig/E7/LAMP-1 genes by PCR. Immunofluorescence was used to determine expression and localization of HPV-16 E7 and Sig/E7/LAMP-1 in recombinant vaccinia-infected cells. The pattern of staining of cells infected with recombinant vaccinia expressing HPV-16 E7 or Sig/E7/LAMP-1 was similar to that

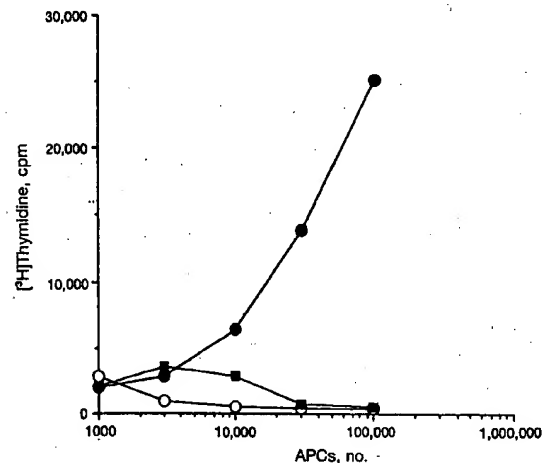


FIG. 4. Proliferation of HPV-16 E7 peptide-primed lymphocytes in response to LB27 APCs transfected with HPV-16 E7, Sig/E7, or Sig/E7/LAMP-1. LB27 cells transfected with Sig/E7/LAMP-1 (solid circles), HPV-16 E7 (open circles), or Sig/E7 (solid squares) were irradiated and coincubated with HPV-16 E7 peptide-specific T cells derived from peripheral draining lymph nodes of CBF1 mice primed with HPV-16 E7 peptide (aa 30–67) in complete Freund's adjuvant. T-cell proliferation was measured by incorporation of [³H]thymidine.

observed with transfection of recombinant plasmids as described above (data not shown), indicating that the vesicular localization of Sig/E7/LAMP-1 product was independent of the expression vector.

We first measured the generation of E7-specific proliferative responses as a measure of T-helper cell activation. Significantly greater lymphoproliferative activity was observed with T cells from C57BL/6 mice infected with Sig/E7/LAMP-1 vaccinia than with wt HPV-16 E7 vaccinia (Fig. 5). The proliferation was HPV-16 E7 peptide specific, since no specific proliferation was observed when pulsed with control peptide (data not shown). Furthermore, CD4-knockout mice did not show significant lymphoproliferative activity compared to that of C57BL/6 mice. These results demonstrated that the endosomal and lysosomal targeting of HPV-16 E7 enhanced presentation of E7 peptides to CD4⁺ T-helper cells.

To measure anti-HPV-16 E7 antibody production, we performed ELISA using plates coated with synthetic HPV-16 E7 peptides or yeast-derived HPV-16 E7 protein (35). Again, the highest titers of anti-HPV-16 E7 antibodies were detected in sera from C57BL/6 mice primed with the chimeric Sig/E7/LAMP-1 expressing vaccinia (Fig. 6). In comparison, anti-HPV-16 E7 antibodies were significantly decreased in sera from CD4-knockout C57BL/6 mice infected with chimeric Sig/E7/LAMP-1 expressing vaccinia (Fig. 6). Finally, we analyzed vaccinia E7 and vaccinia Sig/E7/LAMP-1 immunized mice for E7-specific CTL activity. It was certainly possible that the LAMP-1-mediated targeting of E7 into the MHC class II processing pathway might interfere with processing of MHC class I epitopes. This was not found to be the case since the highest CTL activities were noted in mice that were primed with Sig/E7/LAMP-1 vaccinia (Fig. 7A). Such results were observed with C57BL/6 and BALB/c mice (BALB/c data not shown). The enhanced CTL activity generated by the chimeric Sig/E7/LAMP-1 vaccinia was most likely due to an increase in CD4⁺ T-cell help via improved presentation of MHC class II restricted epitopes, since significant loss of CTL activity was observed in CD4-knockout C57BL/6 mice (Fig. 7B).

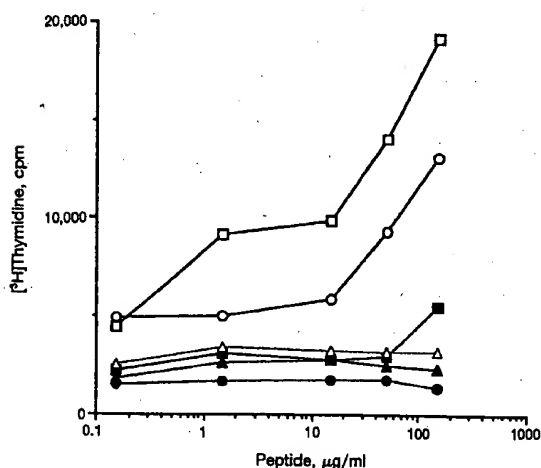


FIG. 5. E7-specific proliferative response of lymphocytes from mice immunized with various recombinant vaccinia. Lymphocytes were prepared from C57BL/6 (open symbols) or CD4-knockout C57BL/6 (solid symbols) mice immunized with 10^7 pfu of Sig/E7/LAMP-1 (squares), HPV-16 E7 (circles), or wt (triangles) recombinant vaccinia virus. Two weeks after infection, splenocytes were isolated and T cells were purified over a nylon wool column. T cells were mixed with naive C57BL/6 or CD4-knockout C57BL/6 splenocytes with decreasing dilutions of E7 30–67 peptide. Proliferation was measured by incorporation of [3 H]thymidine.

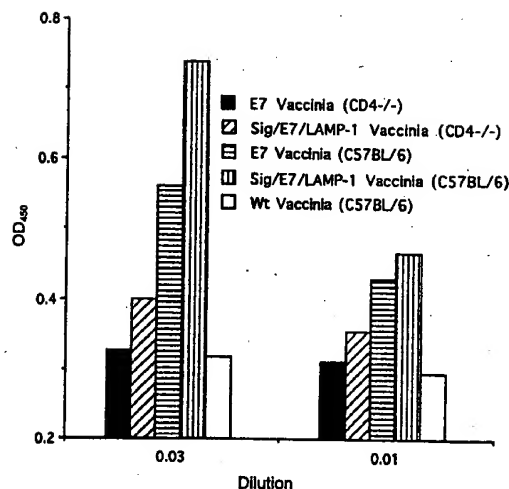


FIG. 6. E7-specific antibody response in C57BL/6 or CD4-knockout C57BL/6 mice immunized with various recombinant vaccinia. C57BL/6 were immunized with wt vaccinia, HPV-16 E7 vaccinia, or Sig/E7/LAMP-1 vaccinia at a dose of 10^7 pfu per mouse. Similarly, CD4-knockout C57BL/6 mice were immunized with HPV-16 E7 vaccinia or E7/LAMP-1 vaccinia at a dose of 10^7 pfu per mouse. Serum samples were obtained from mice 2 wk after infection. ELISA was then performed.

These results clearly demonstrate that the endosomal and lysosomal targeting of HPV-16 E7 enhanced the priming of MHC class II and MHC class I restricted T cells.

DISCUSSION

In this study we have utilized the targeting signal of the lysosomal membrane protein LAMP-1 to direct a model cytoplasmic/nuclear antigen, HPV-16 E7, into the endosomal and lysosomal compartments. This unique approach not only concentrates the antigen in these compartments but also leads to enhanced MHC class II presentation. Furthermore, *in vivo* data suggested that this strategy might enhance the priming of MHC class I restricted T cells as well.

Although our experiments do not directly address the localization of MHC class II peptide loading, the data certainly indicate that antigens directed through the LAMP-1 targeting pathway show enhanced MHC class II restricted CD4⁺ T-cell stimulation. Antigen breakdown may be initiated within proteolytically active endosomal and lysosomal compartments (39–41) and there may exist additional intracellular trafficking pathways for the processed peptides to transfer from the endosomes and lysosomes to compartments where peptides bind with MHC class II molecules.

We also observed enhanced CTL activity in mice infected with E7/LAMP-1 recombinant vaccinia. The ability of LAMP-1-targeted E7 to generate increased CTL activity in mice appears paradoxical since MHC class I restricted epitopes in the wt HPV-16 E7 would be expected to have access to the highly efficient classical MHC class I processing and presentation pathway for nuclear and cytoplasmic antigens. On the other hand, peptides from membrane-associated proteins are also efficiently presented by MHC class I via the transporter associated with antigen processing (TAP)-dependent and TAP-independent pathways (42). Clearly, CTL epitopes from the chimeric Sig/E7/LAMP-1 product are presented on MHC class I molecules. We speculated that the enhanced CTL activity in C57BL/6 mice infected with the chimeric Sig/E7/LAMP-1 vaccinia might be due to an increase in CD4⁺ T-cell help as a result of improved presentation of MHC class II restricted epitopes since similarly

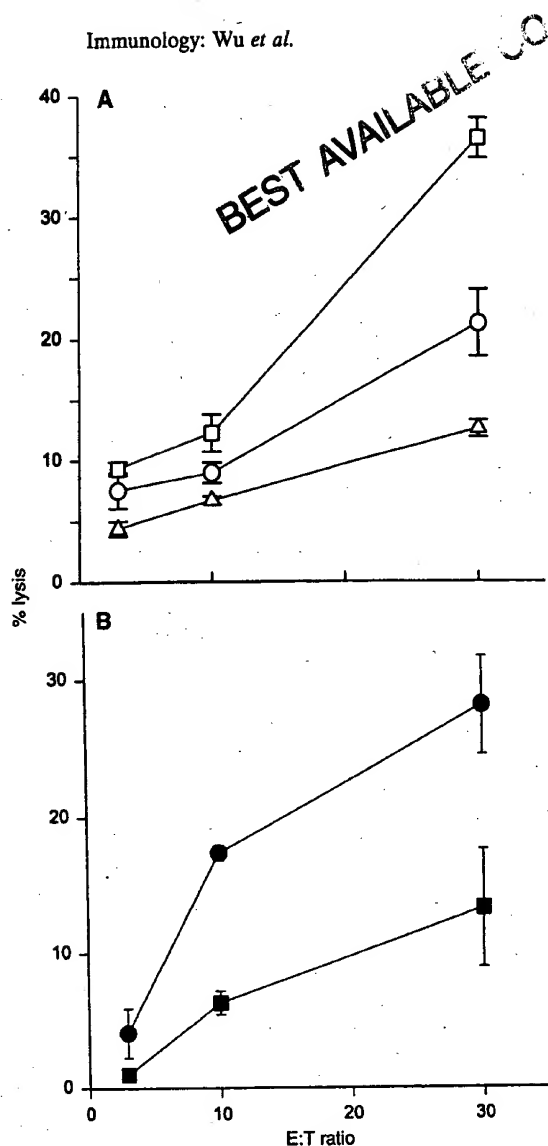


FIG. 7. E7-specific CTL response in C57BL/6 mice immunized with various recombinant vaccinia. (A) C57BL/6 mice were immunized i.p. with 1×10^7 pfu of wt vaccinia (open triangles), HPV-16 E7 vaccinia (open circles), or Sig/E7/LAMP-1 vaccinia (open squares). (B) C57BL/6 (solid circles) or CD4-knockout mice (solid squares) were immunized with 1×10^7 pfu of Sig/E7/LAMP-1 vaccinia. Splenocytes were harvested 2 wk after infection. Splenocytes were stimulated *in vitro* with mitomycin C-treated MC57G-E7, an H-2^b tumor cell, for 6 days before ^{51}Cr release assays were performed. MC57G-E7 or MC57G cells were used as target cells. CD8-blockable lysis was ascertained by incubating splenocytes with rat anti-CD8 mAb (2.43) (38) for 30 min before the ^{51}Cr release assay was performed. Target cell-specific lysis was determined by subtracting each sample's % of MC57G target cell CD8-blockable lysis from its corresponding % of MC57G-E7 cell CD8-blockable lysis. E:T, effector:target. Values are expressed as mean \pm SEM.

treated CD4-knockout C57BL/6 mice significantly lost the enhanced CTL activity.

Our results have a number of implications for MHC class II-dependent antigen presentation and for vaccine design in general. They demonstrate that specific intracellular antigen-targeting strategies can be successfully utilized to enhance the presentation of antigenic epitopes, thereby increasing T-cell stimulation. For such a strategy to effectively enhance vaccine potency, the form of vaccination would require that expression of

the LAMP-1-targeted antigen be endogenous in APCs. Our data with recombinant vaccinia are consistent with the notion that the induction of immune responses involves direct infection of APCs by vaccinia *in vivo*. An additional strategy for vaccine design employing enhanced MHC class II targeting might involve the direct introduction of LAMP-1-tagged antigens into autologous APCs *ex vivo* followed by their reinfusion.

We thank Joseph J. Carter and Denise A. Galloway for providing HPV-16 E7-secreting yeast and Tak W. Mak for providing CD4-knockout mice; Robert J. Kurman, Keerti V. Shah, and Robert Siliciano for helpful discussions and critical review of the manuscript; and Ken-Yu Lin for helping in the preparation of this manuscript. This work was supported by National Institutes of Health Grant 5 po1 34582-01.

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